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ARTICLE

Genotoxicity assessment of ammonia in cultured *Oreochromis niloticus* using RAPD assay

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Abstract Limited efforts have been made to study the genotoxic effects of ammonia in cultured *OOreochromi niloticus*. Therefore, the present study was planned to assess the genotoxic effect of ammonia in cultured *O. niloticus* using random amplified polymorphic DNA (RAPD) assay. Fish was categorized into four groups. The 1st group exposed to 2.5 mg/L of total ammonia nitrogen (TAN) (0.16 NH_3 mg/L), the 2nd exposed to 5.0 mg/L of total ammonia nitrogen (TAN) (0.32 NH_3 mg/L) and the 3rd exposed to 10.0 mg/L of total ammonia nitrogen (TAN) (0.65 NH_3 mg/L), in addition to control group for the treatment period of 6 days. The results revealed that some genes in *O. niloticus* are susceptible to DNA disturbances/mutation as a result of exposure to high concentration of ammonia in water, this clearly indicated using RAPD screening assay.

1. Introduction

Ammonia is the main nitrogenous waste material produced from the catabolism of amino acids, purines and pyrimidines. When fish are reared at high densities, there may be a gradual accumulation of suspended solids and natural catabolites such as ammonia, carbon dioxide and a decrease in dissolved oxygen and an increase in pH, especially if water flow is restricted. In intensive land-based or cage culture systems, there is a continuous production of ammonia depending on the diet used, feeding procedures and general rearing conditions [7,15]. Ambient ammonia may reach high levels especially in integrated culture systems using re-circulated water (up to 70% water re-use), and/or in marine cages or enclosures supplied by water of poor quality caused by accidental or chronic pollution (sewage effluents, industrial or agricultural wastes). In these cases, ammonia acts as a limiting factor for fish growth or survival. A serious type of water pollution, which is primarily originated from agricultural drainage containing fertilizers and growth stimulants as well as from air-borne nitrogenous compounds that comes from automobile exhaust, industrial pollution and ammonia from manure, has been considered the most widespread pollution problem facing aquaculture and freshwater systems [6].

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In many fish species of ornamental and economical values, ammonia toxicity can be a major issue that leads to mass mortality under unfavorable aquacultural conditions [20]. Exposure to such environmental genotoxic agents causes different forms of alterations to exposed aquatic population, which may lead to alteration in both genetic structure and function of ecosystem [3].

DNA alterations include DNA damage (e.g., DNA adducts, breaks), mutations (e.g., point mutations and large rearrangements), and other possible changes (e.g., structural distortion) could be induced by chemical or physical agents following direct and/or indirect interaction with the genomic DNA.

The random amplified polymorphic DNA (RAPD) assay able to detect genotoxin that induces DNA damage and DNA alterations induced by UV, or X-rays [5], heavy metal [8], arsenite [13], quinocetone [12] and to detect genetic instability in tumors [17]. The main advantages of the RAPD method are belonging to its rapidity, applicability to any organism without prior information on the nucleotide sequence, and in the potential detection of DNA damage and mutations. Therefore, RAPD assay was applied to assess the genotoxicity induced by ammonia in Nile tilapia (*Oreochromis niloticus*) in association with acute toxicity.

2. Materials and methods

2.1. Fish and rearing conditions

Hundred *O. niloticus* fish weighting 60.0 ± 10.0 g was obtained from a private fish farm, Sharkya governorate, Egypt. Fish were transported in plastic buckets supplemented with battery aerators to the laboratory of Fish Diseases and Management Department, Faculty of Veterinary Medicine, Cairo University, Egypt. Fish were acclimated in fully prepared glass aquaria supplemented with air pumps containing dechlorinated tap water for 10 days before starting of the experiment. During acclimation, fish were fed on a commercial ration containing 25% protein and dissolved oxygen level was maintained at 6 ± 0.5 mg/L, while water temperature was 22 ± 2 °C. Fish were examined clinically to assure the absence of any abnormalities or external signs according to the methods described by [2]. Feeding was stopped 2 days before the starting of the experiment.

2.2. Experimental design

Eighty *O. niloticus* of almost the same weight and size were categorized into four groups (20 fish each). The 1st group exposed to 2.5 mg/L of TAN (0.16 NH_3 mg/L), the 2nd exposed to 5.0 mg/L of TAN (0.32 NH_3 mg/L) and the 3rd exposed to 10.0 mg/L of TAN (0.65 NH_3 mg/L), in addition to control group at pH 8 and temperature 28°C for six days according to [9]. The experimental protocol followed to that of [1,11,16]. The experimental medium was changed every 24 h with fresh solution. Water was aerated by compressed air to maintain the oxygen concentration at 6 ± 0.5 mg/l. Ammonium chloride (NH_4 Cl) was used as a source of ammonia. Ammonium chloride was analytical reagent grade (Sigma Chemical Company, St Louis, MO, USA). Tissue specimens were collected from different groups at the start and end of the experiment (0 and 6 days) and stored in deep freezer at −80 °C for DNA isolation.

2.3. DNA extraction and RAPD analysis

The DNA was extracted separately by phenol–chloroform from each of the replicates, from toxicant exposed and non-exposed fish. The integrity of extracted genomic DNA was evaluated in 1% agarose gel electrophoresis. A set of 10 OPA oligonucleotide random (10-mer primers) were purchased from Operon Technologies. The RAPD protocol was carried out as described by [10]. Briefly, the RAPD reaction was performed with 100 ng DNA template in a total volume of 25 μL containing 2.5 μL of 10× enzyme assay buffer, 100 μM each of dATP, dCTP, dGTP, and dTTP, 2 nM of random (10 bp) primer, 1.5 mM $MgCl_2$, and 1.5 U of Taq DNA polymerase (Jena Bioscience GmbH- Lobstedter Str. 80, 07749- Jena, Germany). The RAPD protocol consisted of an initial denaturating step of 3 min at 92 °C, followed by 45 cycles at 92 °C for 25 s (denaturation), 36 or 39 °C for 65 s (annealing), and 72 °C for 6 min (extension). Five additional cycles were programmed at 92 °C for 20 s, 36 °C for 75 s, and 72 °C for 7 min. All applications were done in duplicate and an different days. Amplification products were separated electrophoretically in 1.5% agarose gel using Tris-borate-EDTA (TBE) buffer system for 1 h at 115 V. Fractionated bands were detected by ethidium bromide fluorescence under UV light and photographed with a DS-34 Polaroid camera and quantified using Gel-Pro Analyzer (version 3.1) software. The amplifications were carried out in duplicate and on different days for each of the primers used. The presented RAPD profiles helped to define all genetically damaged DNA samples through the indication of the loss or gain of bands, when compared against normal DNA, as 'genetic unstable'. An e.g., of such analysis is shown in Plate 1.

2.4. Analysis of the band pattern of DNA from control and treated fish

The genomic DNA from all samples was obtained and amplified at least twice on different days. The control and treated samples for each experiment were individually amplified but developed together in the same agarose gel. Quantitative analysis was performed by comparing the percentage appearance of each band for the control and treated samples. After eliminating the background, quantitative differences were studied using volume and percentage parameters of the amplified band. Additionally the individual data were grouped together according to the following criteria: bands of high molecular weight (> 800 bp), bands of intermediate molecular weight (500–800 bp) and bands of low molecular weight (< 500 bp).

2.5. Statistical analysis

For statistical analysis, all individual data from control and exposed DNA samples were considered together and analyzed using student'*t*-test.

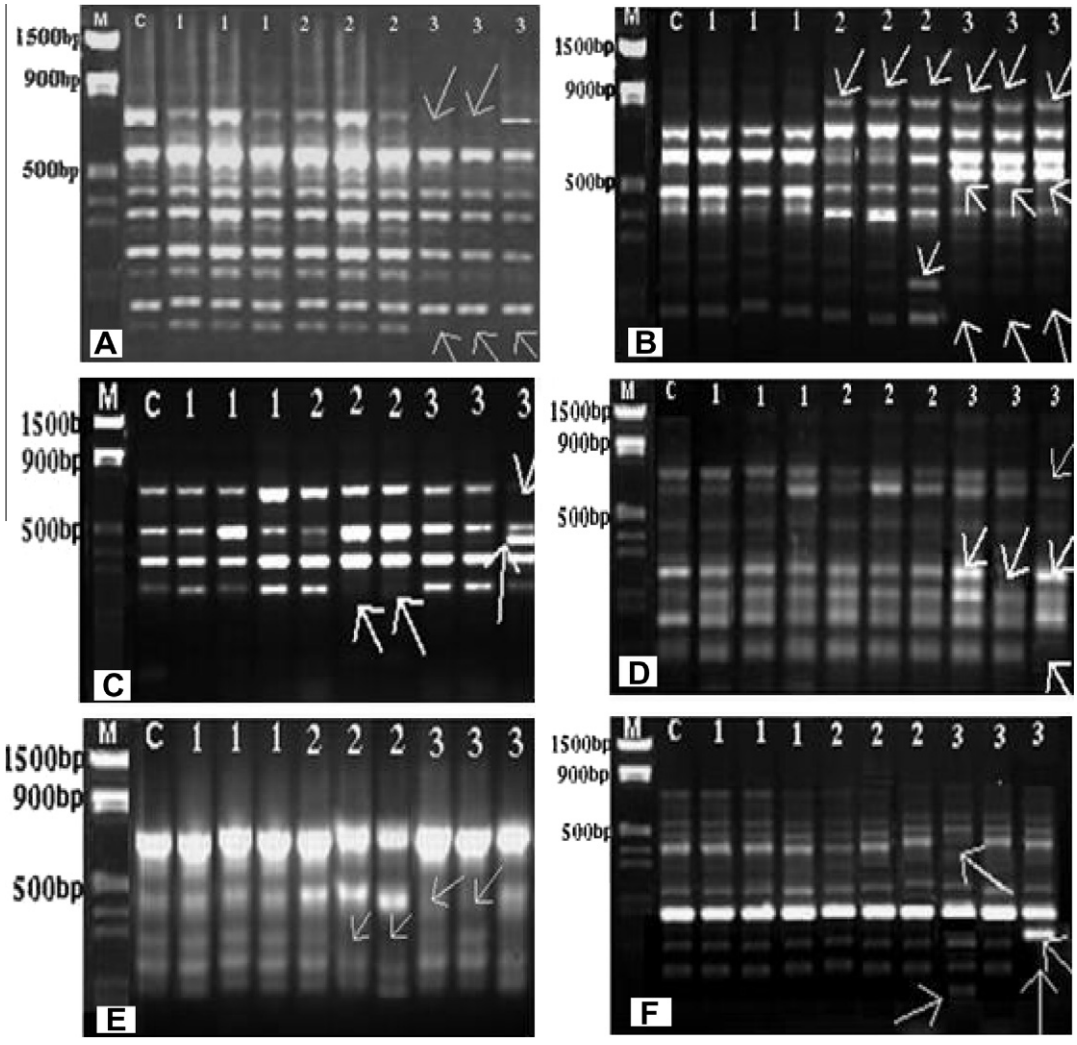

Plate 1 Representative RAPD profiles showing DNA fingerprint patterns with DNA from control, (C) group 1: 2.5 ppm (mg/L) ammonia exposed fish; group 2: 5 ppm (mg/L) ammonia exposed fish and group 3: 10 ppm (mg/L) ammonia exposed fish). Fig. (A): OPA2 results; Fig. (B): OPA4 results; Fig. (C): OPA5 results; Fig. (D): OPA6 results; Fig. (E): OPA8 results and Fig. (F): OPA9 results. Arrows indicate gain/loss differences for amplification products and the size of the amplified fragments (bp).

Table 1 The informative and reproducible primers and their sequences.

Primer name	Sequence
OPA2	5′TGCCGAGCTG 3′
OPA4	5′AATCGGGCTG 3′
OPA5	5′AGGGGTCTTG 3′
OPA6	5′GGTCCCTGAC 3′
OPA8	5′GTGACGTAGG 3′
OPA9	5′GGGTAACGCC 3′

3. Results

The results of the present study indicate that out of the ten used RAPD primers, six primers (Table 1) were informative and generated strong banding patterns in all samples tested.

Furthermore, 4 primers (A,B,C and F) designated OPA2 (5 TGCCGAGCTG 3′), OPA4 (5′AATCGGGCTG 3′),OPA5 (5′AGGGGTCTTG 3′) and OPA 9 (5′GGGTAACGCC 3′) produced reproducible and the most distinguishable banding profiles between non-exposed and exposed samples after at least two independent RAPD assays, as shown in Plate 1.

Similar RAPD-PCR fingerprints were observed in all samples of the control, where about 30% of the specific band of the RAPD pattern was changed by exposed groups either by loss, new bands appearance or band density change specially that of higher concentration treatment (group 3).

In group 1 (2.5 mg/L ammonia treated fish for 6 days), it was noticed that there is no losses or new band appearance but density change in some RAPD-PCR products with two primers (OPA4 and OPA5). The group 2 (5 mg/L ammonia treated fish for 6 days), showed band loss with the primers OPA 5 and OPA 8, in addition new bands were appeared with primer B. Density of some DNA bands was changed in this group using the primers OPA4 and OPA5.

The highest concentration of ammonia in group 3 (10 mg/L ammonia treated fish for 6 days) showed specific loss in the

DNA products amplified with the primers OPA 2, OPA 4, OPA 5, OPA 6 and OPA 8. In addition, appearance of new bands product amplified with primer OPA 4 was observed in the reactions conducted with exposure to ammonia compared to that of the control, as well density of some bands was changed in DNA of this group using the primers OPA 2 and OPA 9.

4. Discussion

To our knowledge, this study represents the first attempt to evaluate genotoxicity associated with acute ammonia toxicity in tilapia fish (*O. niloticus)* and there are no direct comparisons in the same experiments of ammonia and hypoxia tolerance, despite the high likelihood that fish experiencing hypoxia may also experience high (10 mg/L) ammonia levels (and vice versa).

Ammonia was found to be the main acute toxic compounds in leachate as determined by [18] who found that DNA damage was induced in fish exposed to diluted raw leachate and that coincide with the findings in this study.

In the present study, the RAPD-PCR technique was used to determine the potential acute (6 days) ammonia genotoxicity in *O. niloticus*. Different and distinctive finger pattern were obtained from *O. niloticus* DNA under investigation. Primers used in the *O. niloticus* DNA exposed to Ammonia yielded RAPD patterns differ from the control fish. This indicated that DNA from fish exposed to Ammonia created polymorphic regions in the *O. niloticus* genome.

The main changes in the RAPD profiles of the present investigation were the gain or loss of different bands (group 2 and 3) and variation in their intensity (The three groups under investigation). These effects might be due to the structural rearrangements in DNA caused by different types of DNA damages. Appearance of new bands can be explained as a result of different DNA structural changes such as breaks, transpositions and deletions as reported by [14,4].

Estimation about the existence of mutation and structural alterations in *O. niloticus* DNA exposed to ammonia on the bases of DNA patterns could be obtained after RAPD with a set of random primers. The variation in band intensity and disappearance of some bands may correlate with the level of DNA damage after exposure to ammonia, which can change the number of binding sites for Taq polymerase. That due to, the *Taq* DNA polymerase is the most commonly used enzyme in DNA sequencing. As a result, the G track generated during DNA sequencing by these *Taq* polymerases does not terminate prematurely, and higher molecular-mass G bands are detected. Another property of these *Taq* polymerases is that the sequencing patterns produced by these enzymes are remarkably even in band-intensity and peak-height distribution, thus resulting in a significant improvement in the accuracy of DNA sequencing [19].

Although RAPD-PCR provides no direct information on the functional importance of the mutated loci, further analysis of these genetically altered loci may provide suggestive evidence for loci that participate in the *O. niloticus* DNA damage.

Conflict of Interest

The authors declare that there is no conflict of interest.

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